MATERIALS AND METHODS
The details of the materials and methods used in this study for the collection and analysis of data has been described under the following headings:

a) materials used to collect the data
b) methodology used to acquire the data
c) statistical analysis of the data

MATERIALS USED TO COLLECT THE DATA

1) Experimental animal

Male Wistar albino rats, weighing in the range of 250-300 gm, were used for the study. Rats were chosen as experimental animals for the following reasons:

a) easy availability
b) ease of handling
c) rats are polycyclic animals and hence are suitable for this type of study
d) Locus coeruleus is predominantly noradrenergic in case of rats
e) availability of sufficient literature
f) availability of stereotaxic apparatus and atlas for rats.

Experimental rats were obtained from the Central Animal House Facility, JNU. Rats were maintained under 12:12 L:D cycle with food and water ad lib and were kept in polyethylene cages.

2) Equipments

a) Stereotaxic apparatus and brain atlas:

This instrument (Type SR-6, Narishinge Scientific Instrument Lab., Japan) was used for implanting intracerebral stimulating electrodes precisely into the dorso pontine tegmentum locus coeruleus area. Many stereotaxic atlases are available for
precisely implanting the electrodes namely Konig and Klippel (1963), Oswaldo-Cruz and Rocha-Miranda (1968), Pellegrino et al., (1979) etc. The atlas titled 'The rat brain in stereotaxic coordinates' by Paxinos and Watson (1982) was used in this study for implanting the electrodes.

b) **Polygraph:**

It is an instrument used for simultaneous recording of multiple biological signals on chart paper. To record the electrophysiological parameters (EEG, EOG and EMG), a 4 channel Grass Polygraph (Model 79 D) was used. It consists of the following components:

i) polygraph channels

ii) power supply

iii) writer unit (speed regulator): This pushbutton electric shift chart drive provided rapid change in speed. The paper speed could be set at speeds of either 2.5, 5, 10, 25, 50 or 100 mm/sec or mm/min by pressing respective push buttons. In this study, the paper speed was set at 2.5 mm/sec.

iv) Time event marker: It gave tiny marks at either 1, 5 or 60 sec of different amplitudes. The amplitude of marks made at every 1 sec, for instance were all similar but were different in amplitude from those made at either 5 or 60 sec.

Each channel of the polygraph consisted of differential AC preamplifier which required its own driver amplifier for driving the pen. Grass model 7P5 and 7P3 interchangeable preamplifiers were used.

**Differential AC preamplifier** has following specifications:

**input**- a pair of input selector switches, G1 and G2 each with 5 option points for selecting the desired combination of electrodes without disturbing the animal.

**sensitivity**- ranges from 20 μV/cm to 150 mV/cm adjustable by a 9-position switch and
a multiplier knob.

**Frequency response control**: It was used to adjust the low and high frequency responses. 1/2 AMP. LO FREQ. switch selected one of the 5 cut off filters ranging from 0.15 to 10 Hz. The corresponding time constant (in milli seconds) could be read at the opposite end of the indicator knob.

**Calibration switch**: It selected an internal calibration signal which ranged from 10 to 500 on the µv scale and from 1 to 50 on the mv range. The INPUT switch was used to select between the CAL and USE mode. G1 NEG is a red push button, which when pressed, delivered the calibration signal in upward direction (negative) and when released, a downward (positive) signal was obtained. The vertical height of signal in either direction is the deflection for the calibration voltage selected at the amplifications settings used. Once the calibration was done, the USE mode was selected for the recording.

**50 Hz filter**: It selectively rejected waves of line frequency, 50 Hz in this situation.

**Driver amplifier**

It further amplified the power of the signal amplified by the preamplifier, proportionate to the signal, to such a level that the pen could be moved. It consisted of the following adjustment knobs:

i) **Polarity knob**: This knob was used to adjust the movement of the pen, either upwards or downwards with respect to the base line.

ii) **Baseline knob**: It enabled to adjust the pen along a horizontal line.

iii) **50 cycle filter knob**: It eliminated selectively waves of line frequency, 50 Hz in this situation.

iv) **High frequency cut off knob**: It filtered off either the waves of frequency beyond 40, 3, 0.5 kHz and 75, 35, 15, 3, 0.5, 0.1 Hz from the signal depending on the
vii) **Driver sensitivity knob**: This knob provided a continuous adjustment of sensitivity of pen deflection.

The adjustments of different knobs for recording EEG, EMG and EOG in this study were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1/2 Amp. Low Freq.</th>
<th>1/2 Amp High Freq.</th>
<th>Time constant</th>
<th>50 cycle Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEG</td>
<td>1.0 Hz</td>
<td>75Hz</td>
<td>0.1</td>
<td>Out</td>
</tr>
<tr>
<td>EMG</td>
<td>3.0 Hz</td>
<td>75Hz</td>
<td>0.04</td>
<td>Out</td>
</tr>
<tr>
<td>EOG</td>
<td>1.0 Hz</td>
<td>35Hz</td>
<td>0.1</td>
<td>in</td>
</tr>
</tbody>
</table>

c) **Stimulators**:

Two solid-state square wave stimulators (model S44) were used in this study for simultaneous bilateral stimulation. It had following operational controls:

i) **Output on-off-on** lever switch was kept at ON position for the voltage to be delivered at the output.

ii) **Pulses-DC switch** selected between pulses or DC signal at the output.

iii) **Stimulation mode switch** allowed selection between repetitive and single pulses. In repeat position, repetitive pulses were delivered continuously at the set frequency, delay and duration as long as it was kept on. However, in single position, a single pulse was delivered each time the lever switch was activated.

iv) **Stimulation rate control** determined the rate at which pulses were delivered in terms of pulses per second (PPS). The number of pulses per second is equal to the reading on the stimulation rate dial multiplied by the setting of the multiplier switch. The monitor lamp flashed on and off at the same frequency at which stimulation rate
FIG. M1 Diagram showing how voltage/current was monitored during stimulation. Two stimulators (Grass S44) and two stimulus isolation units (Grass SIU) were used but only one of each of those have been shown in the figure for convenience.
dial was set. The rate of stimulation delivered could be adjusted by stimulation rate dial and the multiplier ranged from 0.01 to 1000 pps.

v) delay control- It facilitated the time locking of stimulus to the start of the oscilloscope trace. Delay ranged from 10 μsec to 10 sec. The delay is equal to the setting of the delay dial multiplied by the setting indicated on the multiplier switch.

vi) duration controls determined the length of time (T) in milli- or micro-seconds a pulse of given frequency and voltage was delivered. The duration settings ranged from 10 μsec to 10 sec.

vii) Stimulator output voltage was determined by the volts control knob. The voltage ranged from 0.01 to 150 volts. The voltage is equal to the setting of the volts dial multiplied by the setting indicated on the multiplier switch.

viii) synchronous outputs- The delay-sync-out pulse preceeds the stimulus pulse as selected by the variable delay. It was used everytime when the current was monitored (i.e. every 45 min). This was accomplished by connecting a lead from the sync-out-delay of the stimulator to the external synchronization input of the oscilloscope (Fig. M1; Fig. M4).

The settings used in this study for stimulation are listed below:

<table>
<thead>
<tr>
<th>Stimulation control</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation rate</td>
<td>2Hz</td>
</tr>
<tr>
<td>Duration</td>
<td>300μs</td>
</tr>
<tr>
<td>Stimulation mode</td>
<td>repetitive</td>
</tr>
<tr>
<td>Amperage</td>
<td>200μA</td>
</tr>
</tbody>
</table>

d) Stimulus isolation unit (SIU):

Two Grass stimulus isolation units (SIU5) were used in this study. The isolation units were connected in series with the output of the stimulator (Fig. M1).
These were used to minimize the stimulus artifact. It comprised of the following components:

i) **Direct coupled-capacity coupled**- When the switch was kept in the capacity coupled position, a capacitor was introduced in series with the output and a biphasic pulse appeared at the output.

ii) **Polarity switch**- It determined the polarity of the SIU output.

iii) **Multiplier switch**- This switch was kept at X1 position in both the stimulation units used.

Since voltage stimulator and constant voltage isolation units were used, the stimulating current was monitored approximately every 45 min throughout the experiment.

e) **Oscilloscope**:

This instrument was used for monitoring the stimulus current. The stimulation current was monitored every 45 min during the stimulation period. The monitoring of stimulus current was accomplished by displaying the voltages generated across a known resistance on the oscilloscope. For this purpose, a 1000 ohm resistance was interposed in series in the stimulating circuit. Output from this resistor is fed into one of the channels of the oscilloscope (Fig. M1). Then, the stimulus current can be calculated as follows:

\[ I = \frac{V}{R} \text{ or Stimulus current} = \frac{\text{measured voltage}}{\text{resistance}} \]

This allowed the precise measurement of the magnitude of the stimulus current throughout the experiment and thus a constant current could be delivered to the animal. Since voltage stimulator and constant voltage isolation units were used in this
study, the measurement of the current delivered was necessary. Stimulus current amperage of 200 µA was used in this study. It has been schematically shown in the Fig.M1

f) DCLM5 lesion maker

This instrument was used for electrolytically lesioning the stimulation sites.

At the end of the experiments, rats were anaesthetized and the stimulating plug was connected to the lesion maker. Then, anodal direct current of 500µA was passed for 10 sec.

3) Electrodes

a) EEG electrodes - screw electrodes were used for EEG which were prepared by soldering radio wire to small stainless steel screws (Fig. M2a).

b) EOG electrodes - These were prepared by stripping the insulation at the tip of the flexible radio wires and then making a loop at the stripped end (Fig. M2b).

c) EMG electrodes - these electrodes were same as EOG electrodes except for a hook which was soldered to the looped end as shown in the Fig.M2c.

d) Stimulating electrodes - Two thin (125µm) 40 mm long straight insulated wires, joined along their length keeping the tips separated by about 0.5 mm, were used as bipolar stimulating electrodes (Fig.M2d).

4) Chemicals

The chemicals used in this study, nature of action and their sources are listed below:
FIG. M2  This figure shows: a) EEG electrodes; b) EOG electrodes; C) EMG electrodes; d) Stimulating electrodes.
<table>
<thead>
<tr>
<th>S no.</th>
<th>Chemical</th>
<th>Dose</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>prazosin HCL</td>
<td>3mg/kg</td>
<td>α1 antagonist</td>
<td>Sigma chemicals</td>
</tr>
<tr>
<td>2</td>
<td>propranolol HCL</td>
<td>10 mg/kg</td>
<td>β antagonist</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>yohimbine HCL</td>
<td>0.03 mg/kg</td>
<td>α2 antagonist</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>clonidine HCL</td>
<td>0.1 mg/kg</td>
<td>α2 agonist</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>N-N dimethyl</td>
<td></td>
<td>vehicle for</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>acetamide</td>
<td></td>
<td>prazosin</td>
<td>&quot;</td>
</tr>
<tr>
<td>6</td>
<td>Pentobarbital</td>
<td>35 mg/kg</td>
<td>general anaesthetic</td>
<td>Loba-Chemie Indo-Austral Co.</td>
</tr>
</tbody>
</table>

\[a\] Makela and Hilakivi, 1986; \[b\] Adrien et al., 1985; \[c\] Gaillard et al., 1982; \[d\] Depoortere, 1985; \[e\] Alam and Mallick, 1990

5) Microtome

A rotatory microtome was used to cut paraffin sections of required thickness (40 microns).

METHODOLOGY USED TO ACQUIRE THE DATA

The methodology used can be described under the following subheadings

a) Preparation of animals

b) Recording and collection of data

c) Histological verification of stimulation sites

Preparation of animals

Experimental rats were maintained under 12:12 L:D cycle with food and water ad lib. Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone at the dose of 35 mg/kg. Rats were then fixed in the stereotaxic apparatus for the
implantation of electrodes.

a) Implantation of EEG, EMG and EOG electrodes

Two screw electrodes were implanted bilaterally on the skull for recording EEG. Each of those electrodes was fixed at a distance of 4 mm lateral and 2 mm anterior to bregma. Another screw electrode was implanted on the midline to provide animal ground. EMG electrodes were fixed to the dorsal cervical neck muscles bilaterally whereas EOG electrodes were fixed bilaterally to the muscles near the external canthus.

b) Implantation of stimulating electrodes

The stimulating electrodes were introduced bilaterally up to H 7.0 mm through drill holes made in the skull at stereotaxic coordinates (Paxinos and Watson, 1982) A-9.3 to -10.3 mm and L 1.2 mm to approach the area, locus coeruleus and fixed on skull with dental acrylic. The stimulating and recording electrodes were connected to two separate nine pin female plugs and fixed on the skull with dental acrylic. At least 4 days were allowed for the rats to recover from surgical trauma before recording was started. Second -recovery day onwards the rats were habituated to the recording environment for 4-6 hours each day. Fig. M3 shows a prepared rat with two nine pin female plugs fixed to its head..

c) Recording and collection of data

Bipolar EEG, EMG and EOG were recorded in three separate channels of a Grass polygraph. The experiments were conducted between 9 am-9 pm under similar L:D sequence as that of home cage. Recording set up has been schematically shown in Fig. M4. For convenience, the recording procedure is discussed under the following headings:
FIG. M3  This figure shows a prepared rat with two nine-pin plugs fixed on its head.
FIG. M4  This figure shows the schematic representation of experimental set up
a) pre stimulation (baseline) recording  
b) recording during stimulation of LC  
c) recording during stimulation in adrenoceptor agonist/antagonist treated rats  
d) recording with adrenoceptor agonist/antagonist only  

Pre stimulation (baseline) recording  
A group (Group 1, table 1A) of three rats were prepared without implanting stimulating electrodes for S-W recording only. The baseline S-W was recorded for 22 hours continuously (Table 1A). This was done to compare S-W values during corresponding period of this baseline with that of the experimental (with stimulating electrodes in LC) and control (with stimulating electrodes adjacent to LC) rats. In experimental rats, baseline recording was done on the first day and then the recording was continued along with the stimulation of LC as mentioned below.  

Recording during stimulation of LC  
After the baseline recording, the recording was continued along with the stimulation (bilateral) of LC with low intensity (200μA), low frequency (2 Hz) pulses (300 μs in duration) continuously for eight hours which was followed by post stimulation recording for 4 hours after the cessation of stimulation. In 3 rats, S-W was also recorded the following day without stimulating the LC to observe if the stimulation produced any irreversible change in the normal S-W pattern. Yet, in another control group (Group 5; Table 1A) of 5 rats, the stimulating electrodes were placed away from the LC and S-W recordings continued before, during and after stimulation as mentioned above. Experimental set up is shown in table 1A. Since voltage stimulator and constant voltage isolation units were used, the stimulating current was monitored approximately every 45 min throughout the experiment.  

Recording during stimulation in adrenoceptor agonist/antagonist treated rats
The experimental set up for these experiments is shown in table IB. Baseline sleep-wakefulness recording was done on the first day. On the second day, sleep-wakefulness was recorded simultaneously when the LC was bilaterally stimulated (2 Hz, 200 μA, 300 μsec) for 8 hours which was followed by post stimulation recording for 4 hours as described earlier. After a gap of one day, i.e. on the fourth day, the sleep-wakefulness recording was continued simultaneously in presence (i.p. injection) of either α1 antagonist (prazosin, 3 mg/kg), α2 antagonist (yohimbine, 0.03 mg/kg), β antagonist (propranolol, 10 mg/kg) or α2 agonist (clonidine, 0.1 mg/kg). Prazosin was dissolved in 20% N-N dimethylacetamide (N,NDA) while other antagonists were dissolved in saline. The LC was stimulated (in presence of chemicals) for 8 hours followed by 4 hours post stimulation recording. These chemicals were injected intraperitoneally 5 min. before the start of stimulation. The stimulating current was monitored every 45 min throughout the experiment.

**Recording with adrenoceptor agonist/antagonist only**

After a gap of another day (i.e. on the sixth day), the effect of each of those drugs alone on sleep-wakefulness was studied.

**Histological verification of stimulation sites**

At the end of the experiments, under deep anaesthesia the stimulation sites were lesioned electrolytically by passing anodal direct current 500 μA for 10 sec (Grass DCLM5 lesion maker). Rat brains were perfused intracardially with 20 ml of saline followed by 50 ml of 10% formol-saline containing 2% potassium ferrocyanide. 40 μm sections were cut with the help of microtome. The lesioned sites (Fig. M5) were identified histologically by the presence of prussian blue coloration (Alam and Mallick, 1990; Singh and Mallick, 1996).
FIG. M5  Reconstruction diagram of histological sections through rat brain, as per the atlas of Paxinos and Watson (1982), showing electrolytically lesioned site. Electrode tips were bilaterally located although shown on one side only for convenience. Stimulation of the hatched area reduced REM sleep significantly where as stimulation of the filled areas was ineffective. LC, locus coeruleus; 4V, fourth ventricle; Me5, trigeminal nerve; subC, subcoeruleus nucleus; CGPn, central grey pons; Rpn, raphe pontis nucleus; PnC, pontine reticular nucleus (caudal). The inset shows photomicrograph of histological section of effective lesioned site of stimulation in one of the experimental rats.
DATA ANALYSIS

1) Sleep-wakefulness analysis

Based on EEG, EOG and EMG records sleep-wakefulness stages, every minute, were classified as active wakefulness (AW), quiet wakefulness (QW), slow wave sleep (SWS), deep sleep (DS) and REM sleep (Fig. M6). In short, AW was defined by the presence of desynchronization of the EEG, increased tone with spikes in the EMG and eye movement as evidenced by EOG. During QW the EEG was desynchronized with occasional spindle (<3 spindles per minute or less than 20% of the time spent in spindling), reduced EMG tone and occasional spikes in EMG and EOG. SWS was characterized by the start of high voltage EEG with increasing number of spindles (20-50% of the time spent in spindles), reduced EMG and EOG. DS was characterized by the presence of continuous EEG synchronization, significantly reduced EMG tone and absence of eye movement. REM sleep was classified by the presence of its characteristic signs viz. EEG desynchronization (preceded by DS), EMG atonia accompanied by characteristic waves in the EOG. The term desynchronization refers to low amplitude high frequency waves in EEG whereas synchronization refers to high amplitude and low frequency waves in EEG.

Time spent by the rats in each of those five stages was quantified. The baseline S-W values were compared with that of non-stimulatory control. Mean percent time spent by the rats in each of those states during stimulatory and post-stimulatory periods were compared with respective baseline/ non stimulatory control and stimulatory control values, recorded during identical times, using ANOVA and the significance levels were determined (Table 2). Total REM sleep duration/hr, mean REM sleep frequency/hr, and mean REM sleep duration/episode during both stimulation as well as post stimulation periods were compared to respective baseline
FIG. M6 This figure shows simultaneous polygraphic recording of EEG, EOG and EMG in a rat during different states of sleep-wakefulness-REM.
values. The post stimulatory periods were further divided every hour (PS1, PS2, PS3 and PS4) and each of the values were compared separately with that of the control and respective stimulatory periods. The mean percent time spent by the rats in sleep-wakefulness states during stimulatory period with or without agonist/antagonist and during post-stimulatory periods were compared with respective baseline values by applying ANOVA and the significance levels determined by applying Scheffe post hoc test (Table 3). Similarly, the total REM sleep /hr, mean REM sleep frequency/hr, and mean REM sleep duration/episode during stimulation with or without agonist or antagonist as well as during post stimulation periods were compared with respective baseline values. An hourwise analysis of total REM sleep/hr, REM frequency/hr and REM duration per episode was done during the stimulatory and post stimulatory periods in absence and presence of the drugs.

2) Statistical analysis of data

The data obtained in this study showed a normal distribution which is the essential precondition for many of the parametric tests like T-test, F-test etc. In this study, analysis of variance (ANOVA) has been used.

Analysis of variance (ANOVA)

In ANOVA, all the data are treated at once and a general Null hypothesis of no difference among the means of the various groups is tested. In this simple type of analysis of variance, two types of variations exist: between groups variance and within groups variance. The variation of group mean from the total or grand mean of all the groups is referred as between groups variance. The average variability of the scores within each group is called within groups variance. The analysis of variance is based on the fact that if the groups are random samples from the same population, the two variances, within and between, are unbiased estimates of same population
variance. Significance of the difference of the two types is tested by the use of F-test.

\[
F = \frac{\text{mean square for between groups}}{\text{mean square for within groups}}
\]

The sum of squares between the various groups can be found by the following formula:

\[\sum x^2 = \sum (X - X_t)^2 n\]

Similarly, the within sum of squares for each group can be evaluated by the following formula:

\[\sum x^2 = \sum X^2 - (\sum x)^2 \frac{1}{n}\]

where \(X\) is the mean of each group and \(n\) is the number of samples. This \(F\) value tells us that the means are significant or not. To see where the differences are, a test has been proposed by Scheffe called as Scheffe test (Downie and Heath, 1974). \(F\) values are further computed using the formula:

\[
F = \frac{(X_1 - X_2)^2}{Sw^2(N_1+N_2)/N_1N_2}
\]

where \(X_1\) and \(X_2\) are the means of different groups. \(N_1,N_2\) are the number of samples in each group and \(Sw^2\) is the mean square for within the groups. The \(F\) ratios thus obtained are interpreted by the use of \(F\) table.